

FORM PTO-1390 (Modified)  
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

## TRANSMITTAL LETTER TO THE UNITED STATES

213129US0PCT

DESIGNATED/ELECTED OFFICE (DO/EO/US)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

CONCERNING A FILING UNDER 35 U.S.C. 371

09/926084

INTERNATIONAL APPLICATION NO.  
PCT/JP00/01093INTERNATIONAL FILING DATE  
25 February 2000PRIORITY DATE CLAIMED  
26 February 1999

## TITLE OF INVENTION

NOVEL ENZYME WITH DECOLORIZING ACTIVITY AND METHOD FOR DECOLORIZING DYES BY USING THE SAME

APPLICANT(S) FOR DO/EO/US

SYODA Makoto et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:


1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
- ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☒ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
- ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
- ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

## Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Notice for Consideration of Documents Cited in International Search Report/Notice of Priority/PCT/IB/304  
PCT/IB/308/Drawings (4 Sheets)/Sequence Listing (7 Sheets)

27 AUG 2001

| U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.53)  |              | INTERNATIONAL APPLICATION NO. |   | ATTORNEY'S DOCKET NUMBER  |        |
|---|--------------|-------------------------------|---|---------------------------|--------|
| 09/926084   |              | PCT/JP00/01093                |   | 213129US0PCT              |        |
| 24. The following fees are submitted:   |              |                               |   | CALCULATIONS PTO USE ONLY |        |
| BASIC NATIONAL FEE ( 37 CFR 1.492 (a) (1) - (5) ) :   |              |                               |   |                           |        |
| <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . .                                  |              |                               |   | \$1000.00                 |        |
| <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . . . . .   |              |                               |   | \$860.00                  |        |
| <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . .   |              |                               |   | \$710.00                  |        |
| <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . .  |              |                               |   | \$690.00                  |        |
| <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . .  |              |                               |   | \$100.00                  |        |
| ENTER APPROPRIATE BASIC FEE AMOUNT =  |              |                               |   | \$860.00                  |        |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).  |              |                               |   | \$0.00                    |        |
| CLAIMS  | NUMBER FILED | NUMBER EXTRA                  | RATE  |                           |        |
| Total claims  | 6 - 20 =     | 0                             | x \$18.00   | \$0.00                    |        |
| Independent claims  | 1 - 3 =      | 0                             | x \$80.00   | \$0.00                    |        |
| Multiple Dependent Claims (check if applicable).  |              |                               |   | <input type="checkbox"/>  | \$0.00 |
| TOTAL OF ABOVE CALCULATIONS =   |              |                               |   | \$860.00                  |        |
| <input type="checkbox"/> Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.  |              |                               |   | \$0.00                    |        |
| SUBTOTAL =  |              |                               |   | \$860.00                  |        |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).   |              |                               |   | \$0.00                    |        |
| TOTAL NATIONAL FEE =  |              |                               |   | \$860.00                  |        |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).   |              |                               |   | <input type="checkbox"/>  | \$0.00 |
| TOTAL FEES ENCLOSED =   |              |                               |   | \$860.00                  |        |
|   |              |                               |   | Amount to be: refunded    | \$     |
|   |              |                               |   | charged                   | \$     |
| a. <input checked="" type="checkbox"/> A check in the amount of \$860.00 to cover the above fees is enclosed.   |              |                               |   |                           |        |
| b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.  |              |                               |   |                           |        |
| c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 15-0030 A duplicate copy of this sheet is enclosed.                              |              |                               |   |                           |        |
| d. <input type="checkbox"/> Fees are to be charged to a credit card. <b>WARNING:</b> Information on this form may become public. <b>Credit card information should not be included on this form.</b> Provide credit card information and authorization on PTO-2038. |              |                               |   |                           |        |
| NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.   |              |                               |   |                           |        |
| SEND ALL CORRESPONDENCE TO:   |              |                               |   |                           |        |
| <br>22850<br><br>Surinder Sachar<br>Registration No. 34,423  |              |                               | SIGNATURE<br><br>Norman F. Oblon<br>NAME<br>24,618<br>REGISTRATION NUMBER<br><br>Aug. 27 2001<br>DATE |                           |        |

213129US0 PCT

Rec'd PGT/PTO 26 DEC 2001

#4/  
B

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :  
MAKOTO SYODA ET AL : ATTN: APPLICATION DIVISION  
SERIAL NO: 09/926,084 :  
FILED: AUGUST 27, 2001 :  
FOR: NOVEL ENZYME WITH :  
DECOLORIZING ACTIVITY AND  
METHOD FOR DECOLORIZING  
DYES BY USING THE SAME

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

SIR:

Responsive to the Official Correspondence dated October 23, 2001, Applicants submit herewith a substitute Sequence Listing, a corresponding computer-readable Sequence Listing, and an amendment. Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Page 36 (Abstract), after the last line, beginning on the next page, please delete the original Sequence Listing attached at pages 1 to 7 and replace it with the substitute Sequence Listing attached hereto.

Please amend the specification as shown on the attached marked-up copy following this amendment.

Please replace the paragraph at page 4, line 23, to page 5, line 1, as follows:

--The second aspect of the invention is the enzyme in the first aspect, having the amino acid sequence of SEQ ID NO. 7 in the sequence listing.--

Please replace the paragraph at page 5, lines 2-4, as follows:

--The third aspect of the invention is the gene encoding the enzyme in the first aspect, having the DNA sequence of SEQ ID NO. 8 in the sequence listing.--

Please replace the paragraph at page 16, lines 8-19, as follows:

--After the purified DyP was denatured by ordinary methods, partial hydrolysis thereof using trypsin was performed. Partially digested peptides thus formed were fractionated by HPLC. Consequently, five fragments were recovered. The amino acid sequence of each of the fragments was determined by the Edman method with a protein sequencer. Among the amino acid sequences of the resulting five fragments, the first sequence was Trp Lys. The amino acid sequences of the second and thereafter are shown in the sequence listing, where the second is shown in SEQ ID NO. 1; the third is shown in SEQ ID NO. 2; the fourth is shown in SEQ ID NO. 3 and the fifth is shown in SEQ ID NO. 4.--

Please replace the paragraph at page 16, lines 20-22, as follows:

--Among these amino acid sequences, a partial sequence (SEQ ID NO. 5) of SEQ ID NO. 3 and a partial sequence (SEQ ID NO. 6) of SEQ ID NO. 4 were selected as PCR primers.--

Please replace the paragraph at page 17, lines 11-15, as follows:

--The recombinant plasmid was amplified, by using *E. coli* JM 109 strain. From the resulting plasmid was cutout the coding gene. By a second PCR, the resulting DNA was sequenced (see the positions 1012 to 1181 of SEQ ID NO. 8 in the sequence listing).--

Please replace the paragraph at page 19, lines 1-8, as follows:

--This indicates that pB92 carries the DyP gene. The amino acid sequence of DyP and the nucleotide sequence of the DyP gene, carried in pB92, are shown as SEQ ID NOS. 7 and 8, respectively. In other words, DyP having the amino acid sequence described as SEQ ID NO. 7 in the sequence listing is the enzyme described in the second aspect of the invention, while the gene having the nucleotide sequence described as SEQ ID NO. 8 in the sequence listing is the gene in the third aspect of the invention.--

Please replace the paragraph at page 31, line 22, to page 32, line 8, as follows:

--Homology screening of the DyP gene (SEQ ID NO. 9) was carried out, by using three types of databases (Genebank, EMBL, DDBJ). Consequently, the peroxidase derived from U77073 (Polyporaceae sp.) registered at the Genebank, was screened, which was a gene homologous with DyP. Then, the homology between the two was examined. When regions with high homology were examined, the region at position 407 to position 438 was at the highest homology of 88 %, while the region at position 62 to position 85 was 83 % homologous. For the whole sequence of the gene, only 56 % homology was observed. Additionally, peroxidases with high homology, except for the peroxidase derived from Polyporaceae sp., were never found.--

Please replace the paragraph at page 32, line 18, to page 33, line 8, as follows:

--The classification by Welinder et al. is based on the comparison of highly common sequences in the primary sequence of each peroxidase. In more detail, the classification is practiced by comparing the primary sequences around the His residue proximal to the heme iron and the His and Arg residues distal to the heme iron. Using the sequence comparison table prepared by Welinder et al., the DyP sequence was compared (Fig. 4).

Herein, Fig. 4 includes CCP (Saccharomyces cerevisiae-derived cytochrome C peroxidase) (SEQ ID NO. 10) and ECP (E. coli-derived peroxidase) (SEQ ID NO. 11) as Class I peroxidases. Comparison was done with ARP (Arthromyces ramosus-derived peroxidase) (SEQ ID NO. 12), MnP (manganic peroxidase derived from a fungus of the genus Phanerochaete) (SEQ ID NO. 13) and LiP (Phanerochaete chrysosporium-derived lignin peroxidase) (SEQ ID NO. 14) as Class II peroxidases. Additionally, Class III peroxidase includes TP (Tunip peroxidase) (SEQ ID NO. 15) and HRP (horse radish peroxidase) (SEQ ID NO. 16).--

#### IN THE CLAIMS

Please amend Claims 2 and 3 as follows:

--2. (Amended) An enzyme according to claim 1, having the amino acid sequence of SEQ ID NO. 7 in the sequence listing.

3. (Amended) A gene encoding the enzyme according to claim 1, having the DNA sequence of SEQ ID NO. 8 in the sequence listing.--

#### REMARKS

Claims 1-6 are active in the present application.

Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. Contents of the paper copy of the substitute Sequence Listing and the computer-readable Sequence Listing are identical. Support for all the sequences listed in the substitute Sequence Listing can be found in the present application.

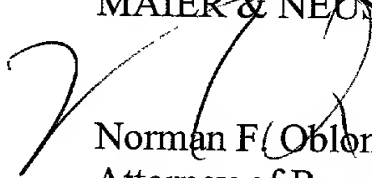
No new matter is introduced by the submission of the substitute Sequence Listing and the computer-readable Sequence Listing.

The specification and claims have been amended to correct typographical errors and give proper reference to sequences in the substitute Sequence Listing. No new matter is believed to be introduced by the amendment.

Applicants submit that this application is in condition for allowance. Early notice to this effect is earnestly solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.

  
Norman F. Oblon  
Attorney of Record  
Registration No. 24,618

Daniel J. Pereira, Ph.D.  
Registration No. 45,518



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**Marked-Up Copy**  
Serial No: 09/926,084  
Amendment Filed on:  
December 26, 2001

IN THE SPECIFICATION

Please amend the specification as follows:

Please replace the paragraph at page 4, line 23, to page 5, line 1, as follows:

--The second aspect of the invention is the enzyme in the first aspect, having the amino acid sequence of [SQ] SEQ ID NO. 7 in the sequence listing.--

Please replace the paragraph at page 5, lines 2-4, as follows:

--The third aspect of the invention is the gene encoding the enzyme in the first aspect, having the DNA sequence of [SQ] SEQ ID NO. 8 in the sequence listing.--

Please replace the paragraph at page 16, lines 8-19, as follows:

--After the purified DyP was denatured by ordinary methods, partial hydrolysis thereof using trypsin was performed. Partially digested peptides thus formed were fractionated by HPLC. Consequently, five fragments were recovered. The amino acid sequence of each of the fragments was determined by the Edman method with a protein sequencer. Among the amino acid sequences of the resulting five fragments, the first sequence was Trp Lys. The amino acid sequences of the second and thereafter are shown in the sequence listing, where the second is shown in [SQ] SEQ ID NO. 1; the third is shown in [SQ] SEQ ID NO. 2; the fourth is shown in [SQ] SEQ ID NO. 3 and the fifth is shown in [SQ] SEQ ID NO. 4.--



Please replace the paragraph at page 16, lines 20-22, as follows:

--Among these amino acid sequences, a partial sequence ([SQ] SEQ ID NO. 5) of [SQ] SEQ ID NO. 3 and a partial sequence ([SQ] SEQ ID NO. 6) of [SQ] SEQ ID NO. 4 were selected as PCR primers.--

Please replace the paragraph at page 17, lines 11-15, as follows:

--The recombinant plasmid was amplified, by using *E. coli* JM 109 strain. From the resulting plasmid was cutout the coding gene. By a second PCR, the resulting DNA was sequenced (see the positions 1012 to 1181 of [SQ] SEQ ID NO. 8 in the sequence listing).--

Please replace the paragraph at page 19, lines 1-8, as follows:

--This indicates that pB92 carries the DyP gene. The amino acid sequence of DyP and the nucleotide sequence of the DyP gene, carried in pB92, are shown as [SQ] SEQ ID NOS. 7 and 8, respectively. In other words, DyP having the amino acid sequence described as [SQ] SEQ ID NO. 7 in the sequence listing is the enzyme described in the second aspect of the invention, while the gene having the nucleotide sequence described as [SQ] SEQ ID NO. 8 in the sequence listing is the gene in the third aspect of the invention.--

Please replace the paragraph at page 31, line 22, to page 32, line 8, as follows:

--Homology screening of the DyP gene (SEQ ID NO. 9) was carried out, by using three types of databases (Genbank, EMBL, DDBJ). Consequently, the peroxidase derived from U77073 (*Polyporaceae* sp.) registered at the Genbank, was screened, which was a gene homologous with DyP. Then, the homology between the two was examined. When regions with high homology were examined, the region at position 407 to position 438 was at the highest homology of 88 %, while the region at position 62 to position 85 was 83 % homologous. For the whole sequence of the gene, only 56 % homology was observed.

Additionally, peroxidases with high homology, except for the peroxidase derived from Polyporaceae sp., were never found.--

Please replace the paragraph at page 32, line 18, to page 33, line 8, as follows:

--The classification by Welinder et al. is based on the comparison of highly common sequences in the primary sequence of each peroxidase. In more detail, the classification is practiced by comparing the primary sequences around the His residue proximal to the heme iron and the His and Arg residues distal to the heme iron. Using the sequence comparison table prepared by Welinder et al., the DyP sequence was compared (Fig. 4). Herein, Fig. 4 includes CCP (Saccharomyces cerevisiae-derived cytochrome C peroxidase) (SEQ ID NO. 10) and ECP (E. coli-derived peroxidase) (SEQ ID NO. 11) as Class I peroxidases. Comparison was done with ARP (Arthromyces ramosus-derived peroxidase) (SEQ ID NO. 12), MnP (manganic peroxidase derived from a fungus of the genus Phanerochaete) (SEQ ID NO. 13) and LiP (Phanerochaete chrysosporium-derived lignin peroxidase) (SEQ ID NO. 14) as Class II peroxidases. Additionally, Class III peroxidase includes TP (Tunip peroxidase) (SEQ ID NO. 15) and HRP (horse radish peroxidase) (SEQ ID NO. 16).--

#### IN THE CLAIMS

Please amend Claims 2 and 3 as follows:

--2. (Amended) An enzyme according to claim 1, having the amino acid sequence of [SQ] SEQ ID NO. 7 in the sequence listing.

3. (Amended) A gene encoding the enzyme according to claim 1, having the DNA sequence of [SQ] SEQ ID NO. 8 in the sequence listing.--

09/926084

JCO3 Rec'd PCT/PTO 27 AUG 2001

213129US-0 PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

#3/a

IN RE APPLICATION OF: :  
MAKOTO SYODA ET AL : ATTN: APPLICATION DIVISION  
SERIAL NO: NEW U.S. PCT APPLN :  
(BASED ON PCT/JP00/01093)  
FILED: HEREWITH :  
FOR: NOVEL ENZYME WITH DECOLORIZING  
ACTIVITY AND METHOD FOR  
DECOLORIZING DYES BY USING THE  
SAME

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS  
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE CLAIMS

Please amend the claims as shown on the marked-up copy following this amendment to read as follows.

6. (Amended) A method for degrading and decolorizing dyes, comprising using an enzyme derived from Geotrichum candidum Dec 1 (FERM BP-7033), which has the following properties:

a) a property to degrade and decolorize dyes;

b) a molecular weight of 60 kDa, by the molecular weight assay using SDS-PAGE;  
c) a molecular weight of 55 kDa, by the molecular weight assay using gel filtration;  
and  
d) pI (isoelectric point) 3.8, by the assay by isoelectric focusing,  
or a microorganism according to claim 5 for degrading and decolorizing dyes.

#### REMARKS

Claims 1-6 are active in the present application. Claim 6 has been amended to remove an improper multiple dependency. No new matter is added. An action on the merits and allowance of claims is solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,  
MAIER & NEUSTADT, P.C.



Norman F. Oblon  
Attorney of Record  
Registration No. 24,618

Daniel J. Pereira, Ph.D.  
Registration No. 45,518



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**Marked-Up Copy**

Serial No: \_\_\_\_\_

Amendment Filed on: \_\_\_\_\_

08/27/01

IN THE CLAIMS

--6. (Amended) A method for degrading and decolorizing dyes, comprising using an enzyme [according to claim 1] derived from Geotrichum candidum Dec 1 (FERM BP-7033), which has the following properties:

a) a property to degrade and decolorize dyes;

b) a molecular weight of 60 kDa, by the molecular weight assay using SDS-PAGE;

c) a molecular weight of 55 kDa, by the molecular weight assay using gel filtration;

and

d) pI (isoelectric point) 3.8, by the assay by isoelectric focusing,

or a microorganism according to claim 5 for degrading and decolorizing dyes.--

4 pnts

## Description

NOVEL ENZYME WITH DECOLORIZING ACTIVITY  
AND METHOD FOR DECOLORIZING DYES BY USING THE SAME

TECHNICAL FIELD

The present invention relates to a novel peroxidase enzyme with high dye degradation activity, the genetic information thereof and a method for degrading and decolorizing dyes by using the same.

BACKGROUND ART

Many of various synthetic dyes discharged from the processes of staining fiber products and from dyestuff production processes are slightly biodegradable substances, involving much difficulty in the degradation thereof in the nature. Because such colored wastewater is hazardous for the nature, regulations over the wastewater have increasingly been tightened.

In the fields of staining industry and dyestuff production industry, wastewater containing dyes has conventionally been treated, mainly by physical or chemical methods such as adsorption, concentration, chemical transformation and incineration. Although these treatment methods are efficient, these methods disadvantageously involve secondary pollution due to the generation of hazardous

byproducts and the discharge of the greenhouse effect gas via high-level energy consumption.

Recently, attention has been focused on a treatment method actively utilizing biotechnology with microorganisms or enzymes, as an alternative of the treatment methods. Several microbial strains capable of degrading dyes and colored substances have already been reported. For example, Phanerochaete chrysosporium as one species of white rot fungus is listed, which is known as one of lignin-degrading fungus.

However, all the dye-degrading microorganisms known so far have an activity to degrade only one or several types of dyes, so the ability of the microorganisms to treat dyes via degradation is naturally limited. Therefore, the development of an efficient method for treating of wastewater containing dyes has been desired.

Some of the present inventors have isolated a microorganism capable of degrading azo type- and anthraquinone type- dyes, namely Geotrichum candidum Dec 1 [which was internationally deposited at the National Institute of Bioscience and Human-Technology, the Ministry of International Trade and Industry, at 1-1-3, Higashi, Tsukuba, Ibaraki, Japan [transferred on February 17, 2000 from the original deposit (FERM P-15348); the accession number was FERM BP-7033] from the nature and have developed a method for degrading and decolorizing a wider range of dyes by microbial treatment

(Japanese Patent Laid-open No. 9-173051).

It has been assumed that the excellent ability of Geotrichum candidum Dec 1 strain to degrade dyes may possibly be based on the peroxidase activity of the fungal strain, but no instance of specific isolation or identification of such enzyme has been found. Hence, the genetic information thereof has absolutely never been elucidated.

The present invention has been attained toward the industrial demand as mentioned above. An object of the invention is to provide an enzyme applicable to more efficient treatment of wastewater containing dyes and a method for degrading and decolorizing dyes by using the enzyme.

The Geotrichum candidum Dec 1 strain exerts an activity to degrade a wide range of dyes and has also prominent enzyme stability. Therefore, the fungal strain per se or after immobilization on an appropriate carrier can be used for degrading dyes.

So as to enhance the industrial applicability, however, the treatment of wastewater containing dyes, particularly dye degradation should essentially be attained in an efficient manner economically.

It is useful for that purpose to use a dye-degrading enzyme owned by said microorganism through isolation and purification rather than to use the microorganism per se, to further elucidate the genetic constitution thereof to realize



the mass production of the enzyme and to use them in combination.

The present inventors have made investigations so as to attain the purpose. Because the novel fungus Geotrichum candidum Dec 1 strain exerts wide decolorizing spectra over various dyes, the inventors have made further investigations with their attention focused on the dye-degrading enzymes produced by the fungus. The inventors have successfully isolated and identified one of the enzymes, elucidated the gene encoding the enzyme and developed a mass expression system of the enzyme.

#### DISCLOSURE OF THE INVENTION

The first aspect of the invention is a peroxidase (sometimes abbreviated as DyP hereinafter) derived from Geotrichum candidum Dec 1 strain (FERM BP-7033), which has the following properties:

- a) a property to degrade and decolorize dyes;
- b) a molecular weight of 60 kDa, by the molecular weight assay using SDS-PAGE;
- c) a molecular weight of 55 kDa, by the molecular weight assay using gel filtration; and
- d) pI (isoelectric point) 3.8, by the assay by isoelectric focusing.

The second aspect of the invention is the enzyme in the first aspect, having the amino acid sequence of SQ ID NO. 7 in

the sequence listing.

The third aspect of the invention is the gene encoding the enzyme in the first aspect, having the DNA sequence of SQ ID NO. 8 in the sequence listing.

The fourth aspect of the invention is an expression plasmid vector comprising the coding gene in the third aspect.

The fifth aspect of the invention is a microorganism transfected with the expression plasmid vector in the fourth aspect.

The sixth aspect of the invention is a method for degrading and decolorizing dyes, which comprises using the enzyme in the first aspect or the microorganism in the fifth aspect for degrading and decolorizing dyes.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the results of the SDS electrophoresis of the enzyme DyP of the invention. In the figure, the left numerical figures show molecular weight, while the upper numerical figures 1 to 5 independently represent the molecular weight marker, crude enzyme solution, the enzyme solution after ion exchange chromatography, the enzyme solution after hydrophobic chromatography and the enzyme solution after ion exchange chromatography, in this order.

Fig. 2 depicts the results of the isoelectric focusing of the enzyme DyP of the invention. In the figure, the right

numerical figures show isoelectric points (pI), while the upper numerical figures 1 to 3 independently represent crude enzyme solution, purified DyP and the isoelectric point marker, in this order.

Fig. 3 is a graph depicting the relation between the enzyme activity of the inventive enzyme DyP and temperature.

Fig. 4 shows comparison between the primary structure in the proximity of the arginine (Arg) residue and the histidine (His) residue positioned at the center of the activity. In the figure, open square ( $\square$ ) shows the position of the proximal Arg residue, while open circle ( $\circ$ ) shows the position of the proximal His residue and closed circle ( $\bullet$ ) shows the position of distal His residue.

#### BEST MODE FOR CARRYING OUT THE INVENTION

The invention is now described in detail hereinbelow.

The peroxidase of the invention is derived from Geotrichum candidum Dec 1 strain. The inventors isolated and purified the enzyme as follows.

[Preparation of culture broth]

According to general methods, Geotrichum candidum Dec 1 strain (FERM BP-7033) was cultured in a liquid culture medium. Any liquid culture medium of any composition can be used, as long as Geotrichum candidum Dec 1 strain can grow in the liquid culture medium. One preferable example is the potato dextrose

culture medium (sometimes abbreviated as PD hereinbelow) manufactured by Difco, Co., Ltd. So as to promote the induction of the intended enzyme, further, dyes may be added to the culture medium.

The cultivating conditions of the fungal strain may satisfactorily be determined in light of the type of the culture medium used. When the PD culture medium is selected, for example, the fungal strain is cultured at 15 to 37 °C, preferably at 30 °C for 3 to 8 days.

The culture broth thus recovered is subjected as a starting material for the purification of the dye-degrading enzyme to the following steps.

[Purification of dye-degrading enzyme]

The dye-degrading enzyme is now to be purified. The purification conditions are not specifically limited. For the purpose of the protection of the enzyme activity against inactivation, the culture broth is preferably handled at a low temperature, particularly in refrigerator.

Specifically, the microorganisms are first separated from the culture broth, from which the supernatant is recovered. In that case, separation processes such as filtration, centrifugation and membrane filtration may satisfactorily be used. Preferably, however, the microorganisms are removed via centrifugation, followed by filtration with glass filter. Contaminating polysaccharides are removed by performing the

combination thereof, to recover a crude enzyme solution at a high purity.

Subsequently, the recovered crude enzyme solution may be subjected to isolation, by using the dye-degrading activity described below as a marker. Prior to such isolation, however, concentration and desalting may be carried out so as to readily enable the separation, to prepare a concentrated crude enzyme solution.

Concentration may be done by methods for general use, for example ultra-filtration, salting-out, and evaporation. Preferably, concentration may be carried out by ultra-filtration. Additionally, desalting may be carried out by dialysis, ultra-filtration and electro-dialysis.

From the recovered concentrated crude enzyme solution can then be isolated the intended dye-degrading enzyme, by using the dye-degrading activity as the marker.

As the method therefor, ion exchange resin column chromatography, hydrophobic column chromatography, gel filtration column chromatography and the like may be used.

One of these column chromatography types or a combination of several types thereof may be used, to collect active fractions to isolate and purify the dye-degrading enzyme.

By the procedures, the inventors recovered the intended purified enzyme. The purified enzyme (205-fold active product) is at a single band by SDS-polyacrylamide gel

electrophoresis (sometimes abbreviated as SDS-PAGE hereinbelow). The enzyme is the dye-degrading enzyme, peroxidase in the first aspect of the invention. The inventors designated the enzyme DyP.

[Properties of the purified dye-degrading enzyme DyP]

The properties of the inventive enzyme DyP purified by the procedures were measured according to the following principles.

First, the molecular weight was measured by SDS-PAGE and gel filtration chromatography.

For the measurement by SDS-PAGE, a commercially available molecular weight standard kit for electrophoresis may be used as the molecular weight standard.

One example thereof includes Combithek manufactured by Boehringer Mannheim Yamanouchi, Co., Ltd. The kit comprises  $\alpha$ -2-macroglobulin (molecular weight of 170 kDa), phosphorylase B (molecular weight of 97.4 kDa), glutamate dehydrogenase (molecular weight of 55.4 kDa), lactate dehydrogenase (molecular weight of 36.5 kDa), and trypsin inhibitor (molecular weight of 20.1 kDa).

As shown in Fig. 1 depicting the results of the measurement, the molecular weight of the inventive enzyme DyP is 60 kDa.

For the assay of the molecular weight of the enzyme by gel filtration chromatography, additionally, the inventors

used Sephacryl S-200 column and the standard molecular weight protein (manufactured by BIO-RAD, CO., LTD.).

Consequently, the molecular weight of the inventive enzyme DyP was assayed as 55 kDa.

Then, the isoelectric point of the inventive enzyme was measured. The isoelectric point was measured by isoelectric focusing.

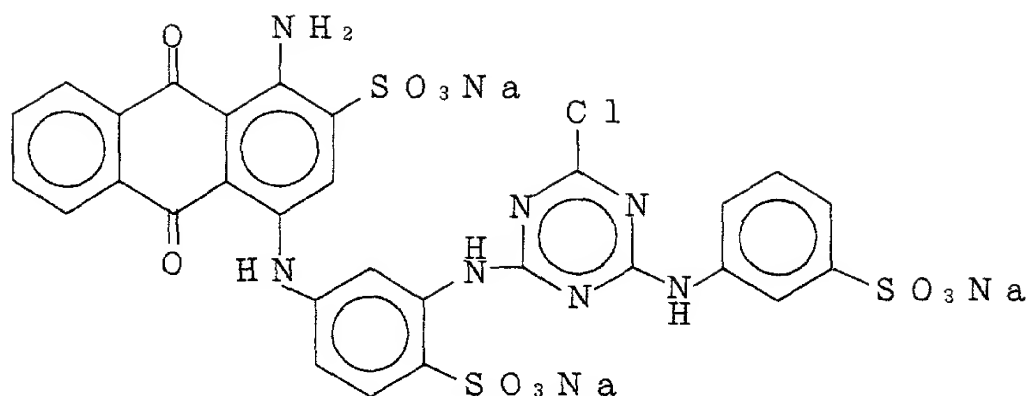
Consequently, the isoelectric point of the enzyme DyP was assayed as pI = 3.8, as shown in Fig. 2.

[Dye-degrading spectrum of dye-degrading enzyme DyP]

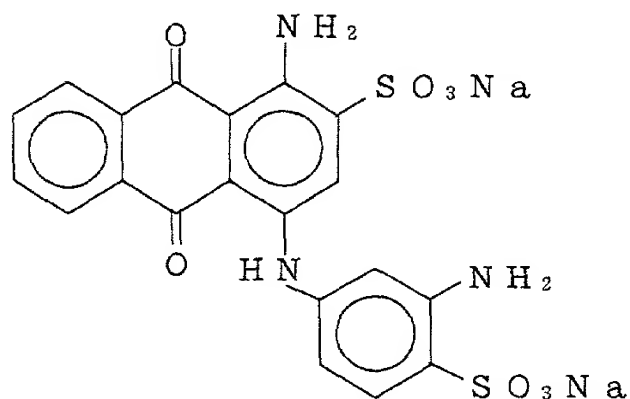
The dye-degrading enzyme DyP of the invention has an enzyme activity over azo type- and anthraquinone type- dyes, in particular, among dyes, and has an ability to degrade and decolorize these pigments.

The anthraquinone type dyes include for example Reactive blue 5, Reactive blue 19 and Reactive blue 114 (all manufactured by Nippon Kayaku Co., Ltd.); 1-amino-4-(3-amino-4-sodium-sulfonoanilino)-2-sodium anthraquinone sulfonate (sometimes abbreviated as AQ-1 hereinafter) and 1-amino-4-methylamino-2-sodium-anthraquinone sulfonate (sometimes abbreviated as AQ-2 hereinafter).

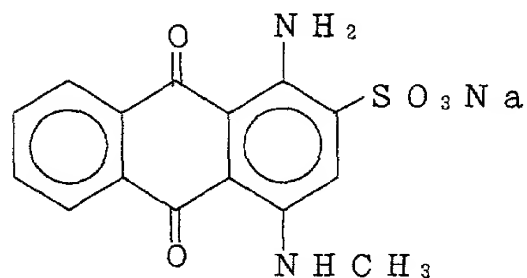
Herein, Reactive blue 5 is the compound represented by the following chemical formula.



AQ-1 is the compound represented by the following chemical formula.



AQ-2 is the compound represented by the following



Further, the azo type dyes include for example Reactive black 5, Reactive red 33, Reactive yellow 2 and Reactive blue 182 (all manufactured by Nippon Kayaku Co., Ltd.).

Other than the dyes, the dye-degrading enzyme DyP has an ability to degrade phenolic compounds such as 2,6-dimethoxyphenol and guaiacol, which are known as substrates for



manganese peroxidase (sometimes abbreviated as MnP hereinafter). As shown in the following examples, however, no effect of manganese compounds added to the reaction solution on the promotion of the enzyme activity is observed.

Alternatively, no reaction of DyP with veratryl alcohol known as a substrate of lignin peroxidase (sometimes abbreviated as LiP hereinafter) is observed.

As described above, surprisingly, DyP exerts substrate specificity different from those of MnP and LiP known so far. Thus, DyP can be said as a peroxidase differing from the known enzymes.

[Optimum reaction temperature of dye-degrading enzyme DyP]

The optimum reaction temperature of the inventive dye-degrading enzyme DyP is around 30 °C, as shown in Fig. 3. The enzyme DyP exerts stable dye-degrading activity within a temperature range of 15 °C to 35 °C. However, the enzyme activity rapidly decreases at a temperature above 35 °C.

[Temperature stability of dye-degrading enzyme DyP]

After the inventive dye-degrading enzyme DyP was stored at a fixed temperature for a fixed period of time, the ratio of the remaining activity was subsequently evaluated. Specifically, a solution of the dye-degrading enzyme DyP in 25 mM citrate buffer was stored at 30 °C or 40 °C for 14 days.

Consequently, the remaining enzyme activity of DyP was at 63 % when DyP was stored at 30 °C; and the activity was at

41 % when DyP was stored at 40 °C.

So as to compare the temperature stability with those of other peroxidases, solutions of individual enzymes in 25 mM citrate buffer were stored at 60 °C for 3 hours, by using a commercially available horse radish peroxidase (manufactured by Wako Chemical Co., Ltd.; sometimes abbreviated as HRP hereinafter) as the control. Subsequently, the remaining enzyme activities were compared to each other.

Consequently, it was shown that 65 % of the activity of the inventive dye-degrading enzyme DyP remained, but only 10 % of the activity of HRP remained.

The results show that the dye-degrading enzyme DyP has greater thermal stability, compared with currently known peroxidases.

The enzyme characteristics of the novel dye-degrading enzyme DyP in accordance with the invention are described hereinabove.

The dye-degrading enzyme DyP of the invention exerts a wider range of dye-degrading activity, compared with any of currently reported dye-degrading enzymes, and has also prominent enzyme stability, as apparently shown in the enzyme characteristics.

In the sixth aspect of the invention, hence, the use of the dye-degrading enzyme DyP enables efficient degradation and decolorization of such dyes.

So as to raise the industrial applicability of the dye-degrading enzyme DyP, essentially, efficient degradation of wastewater containing dyes and dyes should be attained economically.

One example includes a method using DyP after immobilization. The method comprises immobilizing DyP through adsorption or covalent bonding on immobilization carriers, such as ion exchange resin, synthetic polymer gel, naturally originated active charcoal and zeolite, and using the resulting DyP as bioreactor. For creating highly active bioreactor, the method is more useful than the method using the microorganism per se after immobilization.

As a means for more economically producing the enzyme, the gene encoding the intended enzyme is isolated, which is then introduced in a host microorganism capable of expressing the enzyme at a mass scale, so that DyP at a higher purity can be recovered more efficiently in a more stable manner.

Compared with the case of using the microorganism per se, a combination thereof enables the preparation of a bioreactor with a far more excellent cost performance.

[Schema of isolation of gene encoding dye-degrading enzyme DyP]

From the above respects, the inventors carried out the following procedures so as to obtain the genetic information of the DyP of the invention.

The concrete method for isolating the gene is

schematically described below.

First, partial hydrolysis of the inventive dye-degrading enzyme DyP purified by the aforementioned method was carried out by allowing trypsin (manufactured by Wako Chemical Co., Ltd.) to react with the enzyme.

The resulting five types of the partially hydrolyzed fragments were purified. Thereafter the amino acid sequences of the individual fragments were determined, to synthetically prepare the coding gene corresponding to each of the amino acid sequences.

Subsequently, PCR-amplified gene was recovered by PCR using the resulting coding gene as a primer and the cDNA derived from Geotrichum candidum as a template.

The resulting amplified gene was labeled by using the DIG labeling detection kit (manufactured by Boehringer Mannheim, Co., Ltd.).

By general methods using the labeled amplified gene as a probe, plaque hybridization with the Geotrichum candidum Dec 1-derived cDNA library prepared by using lambda phage  $\lambda$ gt10, was performed.

From some hybridized colonies thus recovered were cut out the intended genes, which were then integrated into pUC18 plasmid, for subsequent sequencing. This was used as template for the following PCR.

[Determination of partial amino acid sequence of dye-degrading

enzyme DyP]

So as to prepare a primer for the gene encoding DyP, DyP was purified.

Purification of DyP can be performed by usual methods. The methods include for example a purification method comprising electroblotting from SDS-PAGE gel and a purification method by high-performance liquid chromatography (HPLC).

After the purified DyP was denatured by ordinary methods, partial hydrolysis thereof using trypsin was performed. Partially digested peptides thus formed were fractionated by HPLC. Consequently, five fragments were recovered. The amino acid sequence of each of the fragments was determined by the Edman method with a protein sequencer. Among the amino acid sequences of the resulting five fragments, the first sequence was Trp Lys. The amino acid sequences of the second and thereafter are shown in the sequence listing, where the second is shown in SQ ID NO. 1; the third is shown in SQ ID NO. 2; the fourth is shown in SQ ID NO. 3 and the fifth is shown in SQ ID NO. 4.

Among these amino acid sequences, a partial sequence (SQ ID NO. 5) of SQ ID NO. 3 and a partial sequence (SQ ID NO. 6) of SQ ID NO. 4 were selected as PCR primers.

[Probe preparation]

DNA encoding the two types of amino acid sequences was synthetically prepared by the following method.

By PCR using the resulting primer genes and the cDNA derived from Geotrichum candidum Dec 1 as PCR template, a first-stage gene amplification was practiced. Consequently, new 200-bp primers corresponding to the two primers were recovered.

Both the termini of the primers were subjected to T4 DNA polymerase treatment, to synthetically prepare plasmid-ligation sites. Then, the primers were ligated to the HincII site of pUC 18 as E. coli expression vector, to recover a recombinant plasmid.

The recombinant plasmid was amplified, by using E. coli JM 109 strain. From the resulting plasmid was cut out the coding gene. By a second PCR, the resulting DNA was sequenced (see the positions 1012 to 1181 of SQ ID NO. 8 in the sequence listing).

[Cloning of gene DyP encoding dye-degrading enzyme DyP]

From the Geotrichum candidum Dec 1 strain cultured separately was prepared RNA according to usual methods. From the resulting RNA was purified poly(A)<sup>+</sup>RNA. Subsequently, the recovered poly(A)<sup>+</sup>RNA was used to prepare cDNA with a cDNA kit (manufactured by TaKaRa).

After the recovered cDNA was subjected to ligation with T4 polynucleotide kinase kit, DNA of 1,200 to 2,000 bp was fractionated by electrophoresis.

Further, the DNA was inserted in the EcoRI site of lambda

phage  $\lambda$ gt10, for packaging into the  $\lambda$  phage. The recovered phage was used for infection of E. coli.

Colonies hybridizing with the labeled probe previously prepared were screened. As a result, 11 candidates were obtained.

The results of the measurement described above indicate that DyP of the invention has a molecular weight of 60 kDa at a sugar chain content of 17 %, so the primary amino acid sequence is estimated to be 49.8 kDa. Additionally, the open reading frame of the gene encoding DyP is estimated to comprise 460 amino acids, namely 1380 bp.

Independently using the coding genes of the recovered 11 candidates, PCR was carried out again to evaluate the fragment size of the inserted cDNA. In other words, genes in the proximity of 1380 bp were screened.

Consequently, clone 92 carrying the cDNA of a 1600-bp size was recovered. The cDNA was cut out with BamHI from the recombinant plasmid, which was then integrated in pUC18. The resulting plasmid was designated pB92. It was verified that the clone 92 had a dye-degrading activity based on the dye-degrading enzyme DyP.

[DNA sequence of pB92 gene]

pB92 was sequenced with DNA sequencer. Consequently, it was found that the open reading frame of pB92 comprised 498 amino acids, namely 1494 bp and had a molecular weight of 53,306.





[Purification of dye-degrading enzyme DyP]

150 mL of a PD culture medium (potato-dextrose culture medium, manufactured by Difco, Co., Ltd.) was placed in a 500-mL Erlenmeyer flask, into which 5 ml of the spore suspension of Geotrichum candidum Dec 1 (FERM BP-7033) strain was inoculated. Then, culturing was started. Culturing was continued at 30 °C and 120 rpm for 6 days.

After culturing, the culture broth was cooled to 4 °C and centrifuged at  $7,200 \times g$  for 20 minutes. 4,380 mL of the resulting supernatant was used for the following procedures.

The supernatant was filtered through a glass filter (GC50, manufactured by Toyo Roshi Co., Ltd.), to remove the polysaccharide contained therein.

Then, the filtrate was subjected to ultrafiltration on an ultrafiltration membrane (YM10) manufactured by Amicon, Co., Ltd., to concentrate the filtrate to 60 mL. The concentrate was dialyzed against 25 mM piperazine buffer (pH 5.5) and was then concentrated to 17.2 mL, by using Centriprep10 manufactured by Amicon, Co., Ltd.

The concentrate of 17.2 mL was charged on Super Q 650 M column of  $2.8 \times 6.0$  cm (manufactured by Tosoh Co., Ltd.), which was preliminarily equilibrated with 25 mM piperazine buffer (pH 5.5). Subsequently, the column was rinsed with 200 mL of the same buffer, followed by elution on a linear gradient of 0 to 0.4 M.

[illegible]

[Properties of dye-degrading enzyme]

The molecular weight was determined by SDS-PAGE electrophoresis and gel filtration method.

Consequently, the molecular weight of DyP was assayed as

60 kDa.

For gel filtration, alternatively, Sephacryl S-200 column of 3.1 × 95 cm after equilibration with 25 mM citrate buffer (pH 5.0) was used, together with the standard protein kit manufactured by BIO-RAD Co., Ltd.

Consequently, the molecular weight of DyP was assayed as 55 kDa.

For measurement of isoelectric focusing, a low-pI calibration kit of Multiphor II 2-D for pH 2.5 to pH 6.5, manufactured by Pharmacia, Co. was used. Consequently, the isoelectric point of DyP was assayed as 3.8.

[Assay of dye-degrading activity]

The dye-degrading spectrum of the purified DyP was examined for nine types of dyes and three model compounds. The activity of the purified DyP to degrade these dyes or model compounds was assayed by measuring the degradation rates.

As the dyes, use was made of Reactive blue 5, 19 and 114; AQ-1 and AQ-2; Reactive black 5, Reactive red 33, Reactive yellow 2, and Reactive blue 182.

As the model compounds, additionally, use was made of 2,6-dimethoxyphenol, guaiacol and veratryl alcohol.

The dye-degrading activity was measured as follows.

0.2 to 0.4 mM aqueous hydrogen peroxide was added to a mixture solution of 3 mL of 25 mM citrate buffer (adjusted to the optimum pH for the degradation of each of the dyes)

containing each dye at a fixed pH (30 to 120 ppm) and 1 mL of 1.86 nM DyP solution, to initiate the enzyme reaction. Reaction was performed at 30 °C for a fixed period of time, to assay the reaction rate.

1 U of the dye-degrading activity was defined as the activity to decolorize 1  $\mu$ mole Reactive blue 5 or AQ-2 for one minute. The results are shown in Table 1.

Table 1

(DyP activity to degrade each dye and model compound)

| Color index       | Chromogen | $\lambda_{\text{max}}$ | Optimum pH | Initial concentration (ppm) | Decolorizing activity (ppm/min) |
|-------------------|-----------|------------------------|------------|-----------------------------|---------------------------------|
| Reactive blue 5   | AQ        | 600                    | 3.2        | 100                         | 19.8                            |
| Reactive blue 19  | AQ        | 590                    | 3.2        | 70                          | 13.1                            |
| Reactive blue 114 | AQ        | 620                    | 4.0        | 100                         | 7.8                             |
| AQ-1              | AQ        | 600                    | 3.2        | 60                          | 5.4                             |
| AQ-2              | AQ        | 635                    | 3.0        | 50                          | 19.5                            |
| Reactive black 5  | AZ        | 598                    | 3.2        | 30                          | 0.1                             |
| Reactive red 33   | AZ        | 500                    | 3.2        | 50                          | 0.4                             |
| Reactive yellow 2 | AZ        | 390                    | 3.2        | 100                         | 0.5                             |
| Reactive blue 182 | AZ        | 610                    | 4.0        | 120                         | 20.9                            |

As to 2,6-dimethoxyphenol used as a model compound, alternatively, absorbance at 470 nm was colorimetrically measured, which emerged via oxidation.

That is to say, a mixture solution of 2.79 nM DyP and 0.2 mM 2,6-dimethoxyphenol was reacted with 25 mM citrate buffer

(pH 4.5) containing 0.2 mM hydrogen peroxide.

As to guaiacol, 1 mM guaiacol was used in place of 0.2 mM 2,6-dimethoxyphenol, for absorbance measurement at 465 nm.

The results about the model compounds are shown in Table 2.

Table 2

(DyP activity to degrade model compounds)

| Compound            | Group       | pH  | Initial concentration (mM) | Oxidation rate ( $\Delta$ OD/min) |
|---------------------|-------------|-----|----------------------------|-----------------------------------|
| 2,6-Dimethoxyphenol | phenolic    | 4.5 | 0.2                        | 0.29                              |
| Guaiacol            | phenolic    | 4.0 | 1.0                        | 0.29                              |
| Veratryl alcohol    | nonphenolic | -   | 0.5                        | ND                                |

The results in Table 1 indicate those described below.

The dye-degrading enzyme DyP exerts a high activity to degrade the anthraquinone type pigments. Specifically, the enzyme exerts an excellent degradation activity over Reactive blue 5, Reactive blue 19 and AQ-2, so the enzyme can efficiently degrade these pigments.

Additionally, the enzyme exerts an activity to degrade the azo type pigments. The enzyme efficiently degraded Reactive blue 182, in particular. The enzyme has an ability to degrade other azo type pigments, Reactive black 5, Reactive red 33 and Reactive yellow 2.

This apparently demonstrates that DyP has an action to degrade anthraquinone type pigments and azo type pigments.

Alternatively, the DyP activity over the model compounds is as follows, on the basis of the results in Table 2.

First, 2,6-dimethoxyphenol and guaiacol having phenolic hydroxyl group could efficiently be degraded by DyP. Alternatively, DyP could never degrade veratryl alcohol known as a substrate of lignin peroxidase.

This apparently indicates that DyP has a specifically high enzyme activity over the compounds having phenolic hydroxyl group.

[Optimum temperature of dye-degrading enzyme DyP]

The optimum temperature of DyP was determined by examining the decolorizing (degrading) activity of Reactive blue 5 at a fixed temperature. The results are shown in Fig. 3.

Fig. 3 indicates that DyP exerts a high peroxidase activity within a range of 20 to 35 °C, and also indicates that the optimum temperature is 30 °C.

#### Example 2

[Effect of metal ion on the activity of dye-degrading enzyme DyP]

Each 5 mM ions of calcium, zinc, copper (divalent), potassium, iron (divalent) and sodium were concurrently present in a reaction solution comprising DyP and 100 ppm Reactive blue 5, so as to examine the effects of these metal ions on the relative activity of the DyP enzyme.

The results are shown in Table 3.

Table 3

(Influence of metallic cation on DyP activity to degrade dyestuff)

| Metal ion        | Concentration (mM) | Specific activity (%) |
|------------------|--------------------|-----------------------|
| No addition      | -                  | 100                   |
| Ca <sup>++</sup> | 5                  | 81                    |
| Zn <sup>++</sup> | 5                  | 69                    |
| Cu <sup>++</sup> | 5                  | 75                    |
| K <sup>+</sup>   | 5                  | 81                    |
| Na <sup>+</sup>  | 5                  | 81                    |
| Fe <sup>++</sup> | 0.2                | 50                    |

Table 3 shows that the enzyme exhibited a relative activity of about 80 %, when the ions were added, compared with the case of no addition. Particularly, the divalent iron ion concurrently present at 0.2 mM inhibited the activity at 50 %.

This indicates that the concurrent presence of the metal ions affects adversely the enzyme activity of DyP.

#### Example 3

(Determination of gene encoding DyP and the amino acid sequence thereof)

[Determination of partial amino acid sequence of dye-degrading enzyme DyP]

According to the Laemmli method (Laemmli, U.K. Nature (London), 227, 680-685 (1970)), Geotrichum candidum Dec 1 strain (FERM BP-7033) was subjected to SDS-PAGE, to separate purified DyP.

Subsequently, the DyP was electroblotted on polyvinyl difluoride (sometimes abbreviated as PVDF hereinafter) membrane according to the Towbin method (Towbin, H., Staehelin, T., and Gordon, J. Proc. Natl. Acad. Sci. USA, 76, 4350-4354 (1979)).

The PVDF membrane was treated with Coomassie Brilliant Blue (CBB-250), from which was then cut out solely the membrane corresponding to the stained band portion of the DyP. The band was then transferred in a 1.5-mL test tube. 50  $\mu$ L methanol was added to the test tube, followed by addition of 200  $\mu$ L reductive buffer (buffer, pH 8.5, containing 8 M guanidine hydrochloride salt, 0.5 M Tris buffer, 0.3 % ethylenediaminetetraacetate disodium (EDTA-2Na) and 5 % acetonitrile), for gradual shaking, from which the reductive buffer was removed.

Then, 50  $\mu$ L reductive buffer containing 1 mg dithiothreitol was added onto the protein on the PVDF membrane, which was then left at 25 °C for one hour. After the PVDF membrane was transferred in a 200-mL conical beaker, followed by sequential rinsing individually with 100 mL water for 5 minutes, 100 mL 2 % acetonitrile for 5 minutes and 100 mL 0.1 % SDS for 5 minutes.

Thereafter, the PVDF membrane was transferred into a fresh 1.5-mL test tube, followed by addition of 500  $\mu$ L polyvinylpyrrolidone PVP-40 (sometimes abbreviated as PVP-40 hereinafter) containing 1 mg methionine according to the



Iwamatsu method (Iwamatsu, A. Electrophoresis, 13, 142-147 (1992)), and the resulting mixture was left to stand at ambient temperature for 30 minutes.

After the PVDF membrane was additionally rinsed with 100 mL 10 % acetonitrile solution, it was rinsed three times with 500  $\mu$ L of degradation buffer (100 mM ammonium bicarbonate, 10 mM calcium chloride, pH 7.8). Then the rinse solutions were discarded. Continuously, 500  $\mu$ L of the same degradation buffer except for the content of 1 pmol trypsin was added, and then enzymatic reaction was carried out at 25 °C for 12 hours.

After the oligopeptide eluted from the PVDF membrane into the reaction solution was freeze-dried, the freeze-dried oligopeptide was then dissolved in 100  $\mu$ L degradation buffer, followed by elution on a 0-50 % linear gradient (100 minutes, 0.8 mL/min) of isopropyl alcohol-acetonitrile (7:3 v/v) containing 0.02 % trifluoroacetic acid by HPLC (column: Capcell-Pak C-18, 4.6  $\times$  150 mm), to fractionate individual fractions.

The partially degraded peptides fractionated and purified were determined of their primary structures with a protein sequence system (Shimadzu, PPSQ-21).

[Preparation of cDNA library]

For the purpose of RNA extraction, the culture broth of Geotrichum candidum Dec 1 strain was subjected to centrifugation, to separate 25 mL of hypha of the strain. This

was placed in a centrifuge tube, followed by addition of liquid nitrogen to freeze the hypha, and then it was freeze-dried for 12 hours to recover a powder.

Over the resulting powder of the hypha was again poured liquid nitrogen, to pulverize the hypha, to which was then added 10 mL of a guanidium solution (containing 4 M guanidium isothiocyanate, 20 mM sodium acetate, pH 5.2, 0.1 mM DTT and 0.5 % N-lauroylsarcosine), for homogenization. This was then centrifuged (1,500 × g) to obtain the supernatant.

The resulting RNA was separated by cesium chloride ultra-centrifugation method (Ullrich), followed by fractionation of poly(A)<sup>+</sup> RNA using oligo(dT) cellulose column. The resulting poly(A)<sup>+</sup> RNA was used to synthetically prepare cDNA with a cDNA synthesis kit (TaKaRa; Gulbier-Hoffman).

Into the resulting cDNA was inserted an adaptor (EcoRI-NotI-BamHI), by using a DNA ligation kit. Both the ligated termini were phosphorylated with T4 polynucleotide kinase, to separate cDNA by agarose electrophoresis.

Further, 1200- to 2000-bp cDNA corresponding to the molecular weight of DyP was separated among the resulting cDNAs, to which was then added 8 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA-2Na) to elute the cDNA.

The recovered cDNA fragment was ligated in the EcoRI site of λ phage. This was packaged into λ phage with Gigapack Gold Packaging Extract (Stratagene, La Jolla Calif., USA).

After E. coli NM514 strain was infected with lambda phage  $\lambda$ gt 10 at 37 °C for 15 minutes, the infected strain was overlaid on LB agar medium (bacto-tryptone, 0.5 % bacto-yeast extract, 1 % sodium chloride, 1.5 % agar/1,000 mL), by using 0.7 % agar. The plate was cultured at 37 °C for 12 hours.

[Sequencing of coding gene of dye-degrading enzyme DyP]

Colonies hybridizing with the preliminarily prepared labeled probe were screened. That is to say, by plaque hybridization of the colonies, 11 candidates were consequently selected from the positive cDNA library and DNAs recovered by PCR.

These were ligated with pUC18 plasmid via T4 DNA ligase, for amplification in E. coli JM109 strain.

Because DyP has a molecular weight of 60 kDa at a sugar chain content of 17 %, on the basis of the results of the measurement of the DyP properties, the primary amino acid sequence is estimated to be of 49.8 kDa. Additionally, it is estimated that the open reading frame of the DyP coding gene will comprise 460 amino acids, namely 1380 bp.

Then, the coding genes of the resulting 11 candidates were used for PCR again. Among the inserted cDNAs, a gene around 1380 bp was screened.

Consequently, clone 92 carrying the cDNA of a 1600-bp size was obtained.

By using BamHI, the cDNA was cleaved out of the

recombinant plasmid, which was then inserted in pUC18. The resulting plasmid was designated pB92.

Subsequently, the plasmid DNA was prepared by the alkali extraction method. Both the resulting strands were analyzed and sequenced by a DNA sequencer (Model 4000L, Li-Cor Inc., Lincoln, Neb., USA).

[Expression in a host of different species]

The open reading frame of the pB92 thus recovered comprises 1494 bp, namely 498 amino acids (see SQ ID NO. 7 in the sequence listing). Thus, the molecular weight estimated from the number of the amino acids was 53,306. This indicates that the pB92 carries the DyP gene.

Furthermore, pB92 was transfected into E. coli. The transformant was deposited at the National Institute of Bioscience and Human-Technology, the Ministry of International Trade and Industry, 1-1-3, Higashi, Tsukuba, Ibaraki, Japan. The accession number was FERM BP-7032. The transformant was cultured in L culture medium (0.5 % yeast extract, 0.5 % NaCl, 1.0 % tryptone), and harvested and disrupted, to confirm the DyP activity.

[Comparison with other peroxidase sequences]

Homology screening of the DyP gene was carried out, by using three types of databases (Genbank, EMBL, DDBJ). Consequently, the peroxidase derived from U77073 (Polyporaceae sp.) registered at the Genbank, was screened, which was a gene

homologous with DyP. Then, the homology between the two was examined. When regions with high homology were examined, the region at position 407 to position 438 was at the highest homology of 88 %, while the region at position 62 to position 85 was 83 % homologous. For the whole sequence of the gene, only 56 % homology was observed. Additionally, peroxidases with high homology, except for the peroxidase derived from Polyporaceae sp., were never found.

[Comparison with other peroxidases derived from fungi]

Microbial peroxidase is classified in plant-type peroxidase. Plant-type peroxidases are systematically classified into three classes by Welinder et al. (Welinder, Curr. Opin. Struct. Biol., 2, 388-393 (1992)). According to the classification, peroxidases from prokaryote organisms or eukaryote mitochondria are classified in Class I, while fungal peroxidases are classified in Class II and higher plant-derived peroxidases are classified in Class III.

The classification by Welinder et al. is based on the comparison of highly common sequences in the primary sequence of each peroxidase. In more detail, the classification is practiced by comparing the primary sequences around the His residue proximal to the heme iron and the His and Arg residues distal to the heme iron. Using the sequence comparison table prepared by Welinder et al., the DyP sequence was compared (Fig. 4). Herein, Fig. 4 includes CCP (Saccharomyces cerevisiae-

derived cytochrome C peroxidase) and ECP (E. coli-derived peroxidase) as Class I peroxidases. Comparison was done with ARP (Arthromyces ramosus-derived peroxidase), MnP (manganic peroxidase derived from a fungus of the genus Phanerochaete) and LiP (Phanerochaete chrysosporium-derived lignin peroxidase) as Class II peroxidases. Additionally, Class III peroxidase includes TP (Tunip peroxidase) and HRP (horse radish peroxidase).

Because DyP is derived from the fungus, DyP is classified in Class II. However, it was shown that the proximal Arg residue (at the open square ( □ ) position in the figure) characteristically found in Class II was absent and no homology was found with the sequence in the proximity of the distal His residue (at closed circle ( ● ) position in the figure). This indicates that DyP as a fungus-derived peroxidase has a specific sequence.

#### INDUSTRIAL APPLICABILITY

In accordance with the invention, the peroxidase with high degradation activity of a wide range of dye types as well as the method for degrading the dye types by using the enzyme can be provided. Additionally, the invention provides the genetic information of the enzyme and can supply the enzyme at a large scale on the basis of the information. Thus, the enzyme can be applied to the treatment of wastewater containing dyes

and the like, in the fields of staining industry and the like.

Accordingly, the immobilization of the enzyme as a dye-degrading enzyme can raise the industrial applicability thereof as a bioreactor with higher activity.

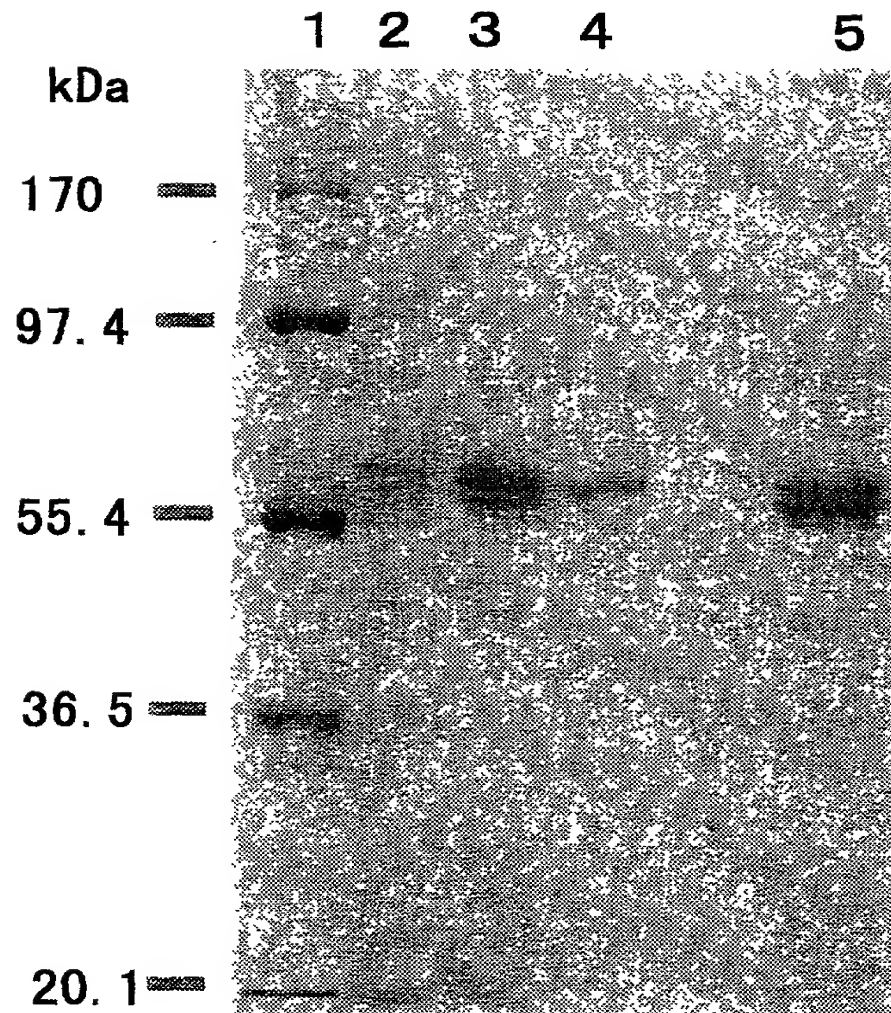
## CLAIMS

1. A peroxidase enzyme derived from Geotrichum candidum Dec 1 (FERM BP-7033), which has the following properties:
  - a) a property to degrade and decolorize dyes;
  - b) a molecular weight of 60 kDa, by the molecular weight assay using SDS-PAGE;
  - c) a molecular weight of 55 kDa, by the molecular weight assay using gel filtration; and
  - d) pI (isoelectric point) 3.8, by the assay by isoelectric focusing.
2. An enzyme according to claim 1, having the amino acid sequence of SQ ID NO. 7 in the sequence listing.
3. A gene encoding the enzyme according to claim 1, having the DNA sequence of SQ ID NO. 8 in the sequence listing.
4. An expression plasmid vector comprising the coding gene according to claim 3.
5. A microorganism (FERM BP-7032) transfected with the expression plasmid vector according to claim 4.
6. A method for degrading and decolorizing dyes, comprising using an enzyme according to claim 1 or a microorganism according to claim 5 for degrading and decolorizing dyes.



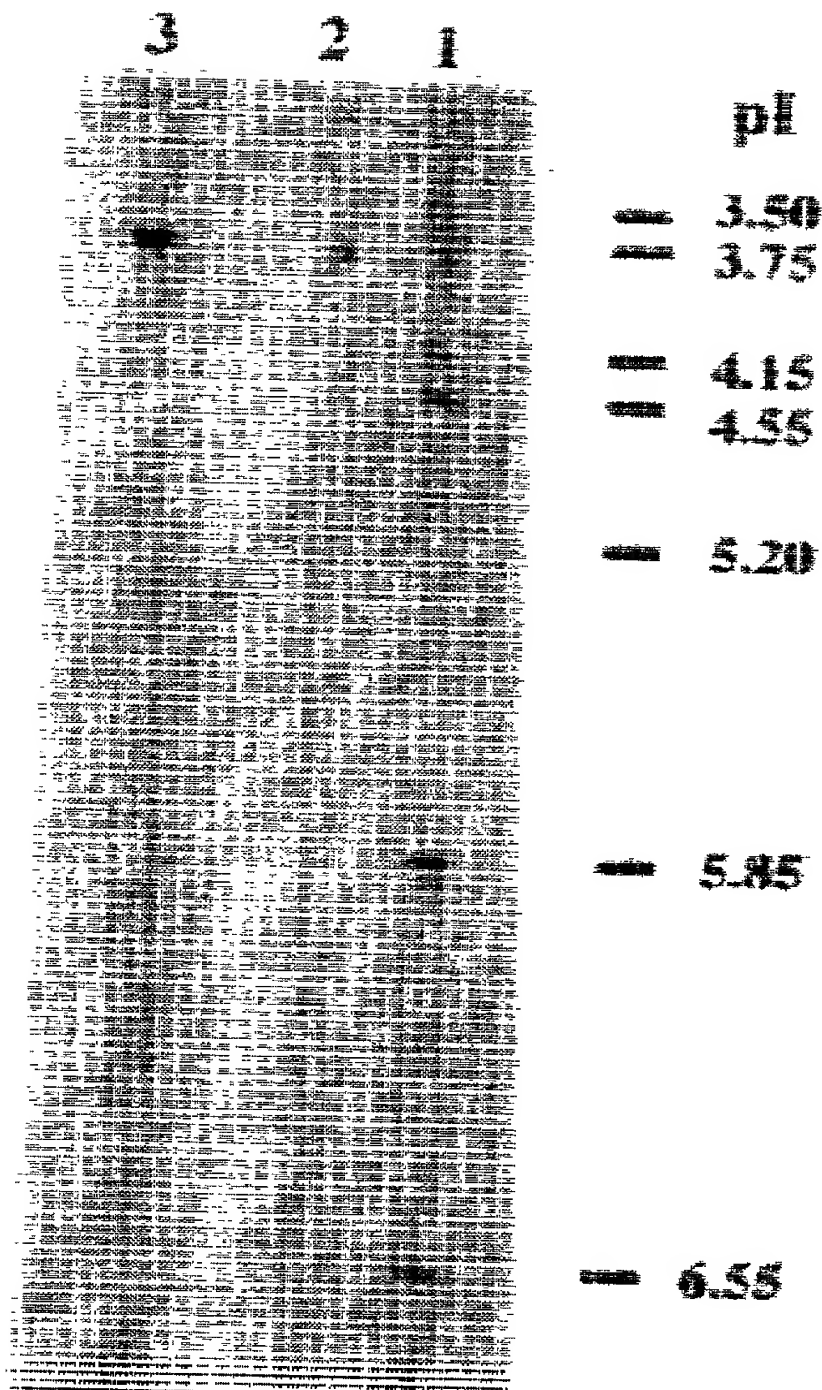
1 / 4

**FIG. 1**



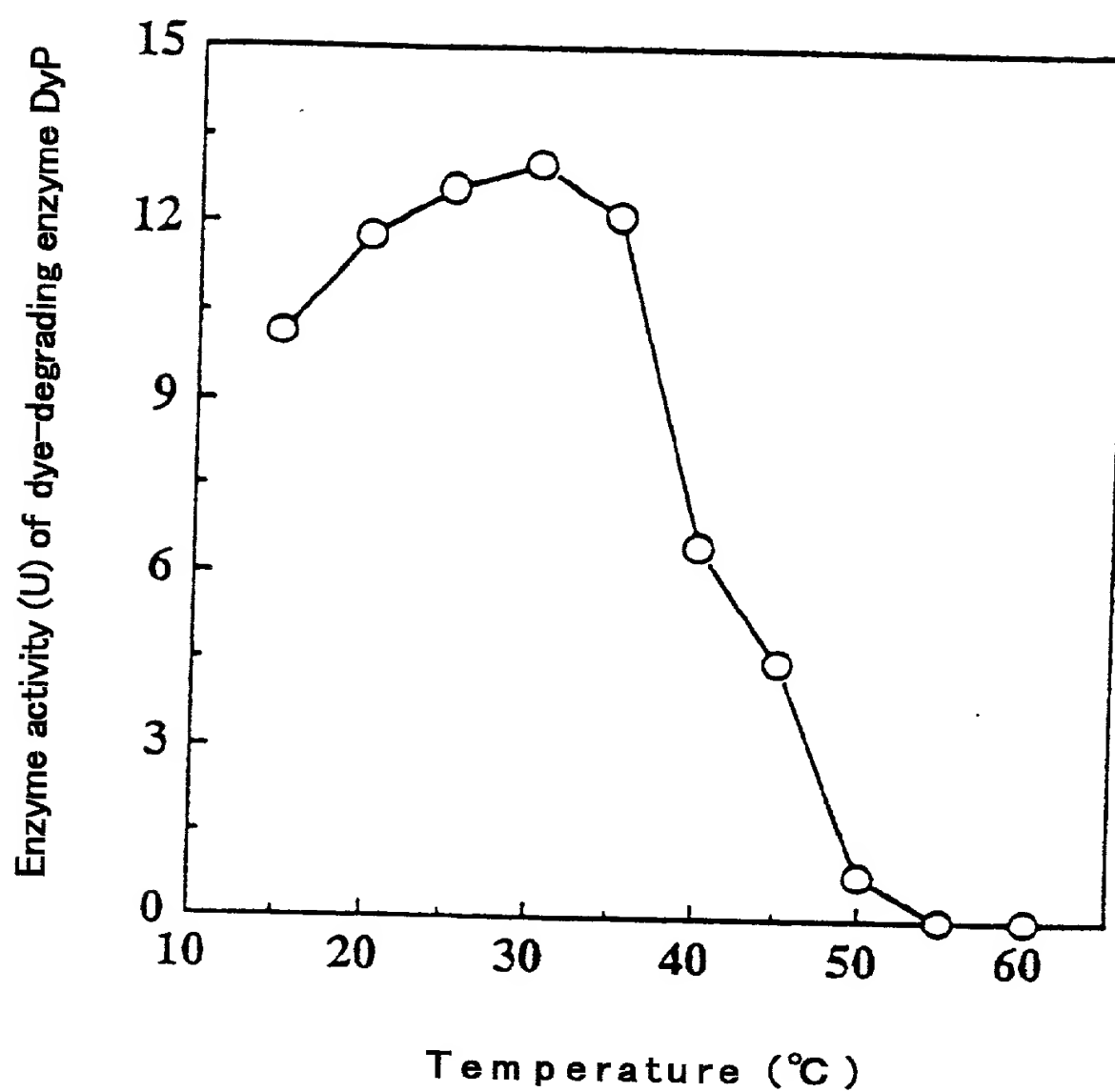
2 / 4

FIG. 2



FOI 2007-03032560

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**FIG. 3**

**FIG. 4**

|   | □   | ○   |     |
|---|-----|-----|-----|
| 43  | 55  | 165 | 177 |
| CCP Gly Pro Val Leu Val Arg Leu Ala Trp His Thr Ser Gly | 109 | 258 | 270 |
| 97  | 59  | 175 | 187 |
| ECP Ala Gly Leu Phe Ile Arg Met Ala Trp His Gly Ala Gly | 49  | 164 | 176 |
| 47  | 50  | 167 | 179 |
| ARP Val Arg Lys Ile Leu Arg Ile Val Phe His Asp Ala Ile | 54  | 165 | 175 |
| 37  | 45  | 159 | 171 |
| MnP Ala His Glu Val Ile Arg Leu Thr Phe His Asp Ala Ile | 45  | 161 | 173 |
| 38  | 45  | 161 | 173 |
| LiP Ala His Glu Ser Ile Arg Leu Val Phe His Asp Ser Ile | 45  | 161 | 173 |
| 42  | 45  | 161 | 173 |
| DyP Gln Ala Pro Leu Pro Thr Leu Thr Gln His Thr Thr Glu | 45  | 161 | 173 |
| 33  | 45  | 161 | 173 |
| TP Gly Ala Ser Ile Leu Arg Leu Phe Phe His Asp Cys Phe  | 45  | 161 | 173 |
| 33  | 45  | 161 | 173 |
| HAP Ala Ala Ser Ile Ile Arg Leu His Phe His Asp Cys Phe | 45  | 161 | 173 |

200 Yasushi SUGANO  
NAME OF SECOND JOINT INVENTOR

Yasushi Sugano  
Signature of Inventor

August 7, 2001

Date

300 Hidetoshi KUBOTA  
NAME OF THIRD JOINT INVENTOR

Hidetoshi Kubota  
Signature of Inventor

August 7, 2001

Date

NAME OF FOURTH JOINT INVENTOR

Signature of Inventor

Date

NAME OF FIFTH JOINT INVENTOR

Signature of Inventor

Date

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Kanagawa 222-0022 JAPAN

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Saitama 350-0214 JAPAN JPX

Citizen of: JAPAN

Post Office Address: c/o Bioscience Labs.,  
Meiji Seika Kaisha Ltd.  
3-1, Chiyoda 5-chome, Sakado-shi,  
Saitama 350-0214 JAPAN

Residence: \_\_\_\_\_

Citizen of: \_\_\_\_\_

Post Office Address: \_\_\_\_\_

Residence: \_\_\_\_\_

Citizen of: \_\_\_\_\_

Post Office Address: \_\_\_\_\_

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

|                      |               |
|----------------------|---------------|
| _____                | _____         |
| (Application Number) | (Filing Date) |
| _____                | _____         |
| (Application Number) | (Filing Date) |


We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

| Application Serial No. | Filing Date | Status (pending, patented, abandoned) |
|------------------------|-------------|---------------------------------------|
| _____                  | _____       | _____                                 |
| _____                  | _____       | _____                                 |
| _____                  | _____       | _____                                 |

And we (I) hereby appoint: Norman F. Oblon, Registration Number 24,618; Marvin J. Spivak, Registration Number 24,913; C. Irvin McClelland, Registration Number 21,124; Gregory J. Maier, Registration Number 25,599; Arthur I. Neustadt, Registration Number 24,854; Richard D. Kelly, Registration Number 27,757; James D. Hamilton, Registration Number 28,421; Eckhard H. Kuesters, Registration Number 28,870; Robert T. Pous, Registration Number 29,099; Charles L. Gholz, Registration Number 26,395; Vincent J. Sunderdick, Registration Number 29,004; William E. Beaumont, Registration Number 30,996; Steven B. Kelber, Registration Number 30,073; Robert F. Gnuse, Registration Number 27,295; Jean-Paul Lavalleye, Registration Number 31,451; Timothy R. Schwartz, Registration Number 32,171; Stephen G. Baxter, Registration Number 32,884; Martin M. Zoltick, Registration Number 35,745; Robert W. Hahl, Registration Number 33,893; Richard L. Treanor, Registration Number 36,379; Steven P. Weihrouch, Registration Number 32,829; John T. Goolkasian, Registration Number 26,142; Marc R. Labgold, Registration Number 34,651; William J. Healey, Registration Number 36,160; Richard L. Chinn, Registration Number 34,305; Steven E. Lipman, Registration Number 30,011; and Jacques M. Dulin, Registration Number 24,067; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Makoto SYODA  
NAME OF FIRST SOLE INVENTOR

  
Signature of Inventor

August 7, 2001  
Date

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JPX  
JAPAN

# Declaration, Power Of Attorney and Petition

Page 1 of 3

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

NOVEL ENZYME WITH DECOLORIZING ACTIVITY AND METHOD FOR

DECOLORIZING DYES BY USING THE SAME

the specification of which

☐ is attached hereto.

☐ was filed on \_\_\_\_\_ as

Application Serial No. \_\_\_\_\_

and amended on \_\_\_\_\_.

☒ was filed as PCT international application

Number \_\_\_\_\_ PCT/JP00/01093

on \_\_\_\_\_ February 25, 2000,

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

| Application No. | Country | Day/Month/Year | Priority Claimed  |
|-----------------|---------|----------------|---|
| 50562/1999      | Japan   | 26/02/1999     | <input checked="" type="checkbox"/> Yes <input type="checkbox"/> No |
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09/926084

JCO3 Rec'd PCT TO 27 AUG 2001

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25

30

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35

40

45

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# SEQUENCE LISTING

<110> SYODA, MAKOTO  
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KUBOTA, HIDETOSHI

<120> NOVEL ENZYME WITH DECOLORIZING ACTIVITY AND METHOD FOR DECOLORIZING DYES  
BY USING THE SAME

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Val Phe Pro Gly Gln Ala Val Val Pro Pro Gly Ile Ile Leu Thr Gly  
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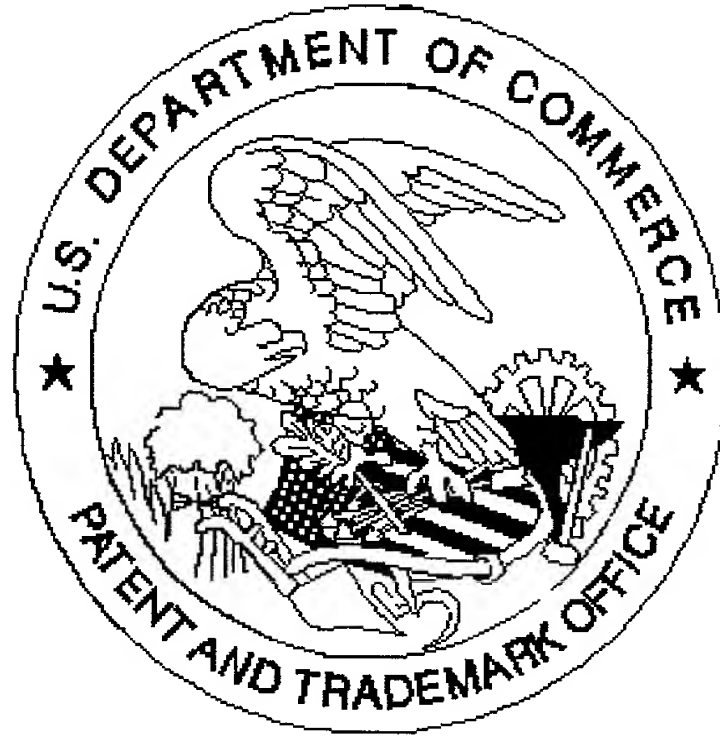
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